Eur J Clin Chem Clin Biochem
1995; 33:775–783

Differential Induction of Peroxisomal Enzymes by Hypolipidaemics in Human (HepG2) and Rat (MH1C1) Hepatoma Cell Lines[1])

By *Herbert Stangl*[1], *Werner Kovacs*[1], *Peter Böck*[2] and *Karl Kremser*[1]

[1] *Institut für Medizinische Chemie der Universität Wien, Wien, Austria*
[2] *Institut für Histologie der Veterinärmedizinischen Universität Wien, Wien, Austria*

(Received May 22/July 25, 1995)

Dedicated to Professor Dr. Erich Kaiser on the occasion of his 70th birthday

Summary: Human (HepG2) and rat (MH1C1) hepatoblastoma cells were incubated with different concentrations of the hypolipidaemics cetaben, clofibrate and thyroxine. The enzymatic activities of catalase, peroxisomal bifunctional enzyme, succinate dehydrogenase, and 3-oxoacyl-CoA thiolase were measured. In order to determine the point of regulation of the enzymatic activities Northern and Slot blot experiments with probes for peroxisomal bifunctional enzyme, catalase and fatty acyl CoA oxidase were performed on total RNA. Catalase activity was enhanced in HepG2 cells treated with 3 mmol/l clofibric acid to 135% of control and the mRNA value to 2.6 fold, whereas in cetaben treated cells the enhancement (up to 119% of control) was less pronounced. In MH1C1 cells catalase activity was not changed by any of the drugs. The activity of the peroxisomal bifunctional enzyme was not affected in HepG2 cells by clofibric acid and cetaben, whereas the mRNA level was elevated to 2.3 fold by 10 μmol/l cetaben. At high concentrations of cetaben all enzyme activities were decreased in both cell lines due to its high cytotoxicity. Our data show that, due to the differences in the genomic organisation, the regulation of the enzyme activities is different in human and rat, but the results from the human and rat hepatoblastoma cells correlate with the findings in whole man and rat, so that a human in vitro system is more suitable for pharmacological tests. These results suggest that the human hepatoma cell line HepG2 may be a useful model system for studies of the influence of hypolipidaemics on the peroxisomal enzyme system.

Introduction

Peroxisomes are ubiquitous subcellular organelles which play important metabolic roles in nearly all animal cells (1). They contain hydrogen peroxide-producing oxidases like fatty acyl-CoA oxidase[2]) and catalase[2]) for the detoxification of hydrogen peroxide (1). The amount of the peroxisomal fraction in rodent liver cells increases several-fold upon treatment with a variety of structurally different compounds such as hypolipidaemic drugs, phthalates, thyroxine and fatty acids (2). This process, known as peroxisome proliferation, increases the cellular capacity of the β-oxidation of very long-chain fatty acids by the induction of peroxisomal enzymes (2). Nevertheless, the response in human and non-human primates is much less pronounced than that observed in the rat and mouse (for review see (3, 4)). Fibric acid derivatives are a well known group of hypolipidaemic drugs, mainly used for the treatment of hyperlipidaemia in human (5).

[1] Supported by "Medizinisch-Wissenschaftlicher Fonds des Bürgermeisters der Bundeshauptstadt Wien"
[2]) Enzymes:
(Fatty) acyl CoA oxidase, acyl-CoA : oxygen oxidoreductase (EC 1.3.3.-)
Catalase, hydrogen-peroxide : hydrogen-peroxide oxidoreductase (EC 1.11.1.6)
Peroxisomal bifunctional enzyme, enoyl-CoA hydratase, and 3-hydroxyacyl-CoA dehydrogenase (EC 4.2.1.17 and EC 1.1.1.35)
Succinate dehydrogenase, succinate : (acceptor) oxidoreductase (EC 1.3.99.1)
(3-oxoacyl-CoA) Thiolase, Acyl-CoA acetyl-CoA acyltransferase (EC 2.3.1.16)

The model fibrate clofibric acid (2-(4-chlorophenoxy)-2-methylpropionic acid), one of the best characterized hypolipidaemic agents, is known to produce a profound hepatomegalic response, including proliferation of hepatic endoplasmic reticulum, mitochondria and peroxisomes after chronic treatment of rodents (6).

A different class of chemicals, lowering the concentration of lipids in blood and also noted for peroxisome proliferative efficiency, is represented by several alkylaminobenzoic acids (7). *Chandoga* et al. (8) have reported on the effect of cetaben (sodium *p*-hexadecyl aminobenzoate), a representative of this class of non fibrate peroxisome proliferators, on the activities of peroxisomal enzymes in liver and kidney of rats, but little is known about their effects in humans.

Like clofibrate, thyroid hormones lead to proliferation of liver peroxisomes (9). Furthermore, thyroid hormones increase the peroxisomal fatty acid β-oxidation, presumably by inducing acyl-CoA oxidase (10), the rate limiting enzyme of this pathway (11). Various models describe the modulation of thyroid hormone action by differential mechanisms via homo- or heterodimerization with nuclear receptors (12).

The regulation of the induction of peroxisomal enzymes seems to be different in different species (4, 13). In rat a single acyl-CoA oxidase gene is present, which gives rise to two different mRNAs by differential splicing (14). *Schepers* et al. (15) identified two different fatty acyl-CoA oxidases in rat liver, one of which is inducible by clofibrate. Only one gene for enoyl-CoA hydratase : 3-hydroxyacyl-CoA dehydrogenase has been identified in rat (16), which is inducible. Finally, two genes for peroxisomal 3-oxoacyl-CoA thiolase are present in rat; one is inducible by clofibrate (17). Thus, entirely different mechanisms are responsible for the induction of individual peroxisomal β-oxidation enzymes in rodents by clofibrate, e. g.

(a) enhanced synthesis of a specific mRNA formed by alternative splicing of the transcript of a single gene (acyl-CoA oxidase), or

(b) enhanced transcription of a separate gene (thiolase), or

(c) enhanced transcription of a single gene (enoyl-CoA hydratase : 3-hydroxyacyl-CoA dehydrogenase) (13).

The situation in human appears to be different, as there is only a single gene for peroxisomal 3-oxoacyl-CoA thiolase (18), and also only one for fatty acyl-CoA oxidase, with properties similar to those of the non-inducible isoform in rat liver (19). This may be related to the fact that compounds like clofibrate do not induce peroxisomal β-oxidation enzymes in primates (20). In this respect it will be of interest to see if the response in primates correlates with the response in human.

The aim of the present study was to show the difference in the regulation of induction of peroxisomal enzymes by peroxisomal proliferators in comparison of rats and human. A rat and a human hepatoma cell line, MH1C1 and HepG2, respectively, were selected as model systems. In our approach we were able to demonstrate the differential regulation of the induction of peroxisomal enzymes as a response to the application of hypolipidaemic drugs on cell cultures of human and rat liver cells.

Materials and Methods

Cell culture

HepG2 cells, derived from a human hepatoblastoma, were obtained from the American Tissue Culture Collections (Rockville, USA) and grown in *Dulbecco*'s Modified Eagles Medium (GIBCO/BRL; Paisly, UK) supplemented with foetal bovine serum (GIBCO/BRL; Paisley, UK), volume fraction 0.1. The MH1C1 cell line, derived from the Morris 7795 rat hepatoma, was obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) and grown in *Dulbecco*'s Modified Eagles Medium supplemented with foetal bovine serum, volume fraction 0.15. All media were supplemented with 100×10^3 IU/l penicillin G and 100 mg/l streptomycin. The cells were kept at 37 °C in a humidified atmosphere of 10% CO_2 and 90% air (isobaric).

For all experiments cells were plated at a density of 2×10^6 cells per Petri dish (diameter 100 mm) and grown to confluence. All incubation media were prepared by adding the appropriate volume of a stock solution of test compound. Clofibric acid (stock solution: 1 mol/l) was dissolved in dimethylsulphoxide. Cetaben (25 mmol/l stock solution) was suspended in phosphate buffered saline (8 g/l NaCl, 0.2 g/l KCl, 0.2 g/l KH_2PO_4, 0.1 g/l $CaCl_2$, 0.1 g/l $MgCl_2 \cdot 6H_2O$, 2.9 g/l $Na_2HPO_4 \cdot 12H_2O$, pH = 7.2).

Triiodothyronine (50 mmol/l stock solution) was prepared in 0.01 mol/l NaOH. Incubation media were sonicated for 10 min at room temperature before use. Treatment of the confluent monolayers was commenced by replacing the culture media with incubation media supplemented with the test compounds. Incubations were carried out for 24 hours. The monolayers were washed two times with phosphate buffered saline. The cells were detached by means of trypsin-EDTA (0.5 g/l trypsin, 0.2 g/l EDTA) for 5 min at 37 °C. Cell suspensions were sedimented for 5 min at 300 *g*, resuspended, centrifuged again and suspended in ice cold phosphate buffered saline.

Enzyme assays

For the determination of catalase activity, cells were incubated with 10 g/l Triton X-100 for 2 min prior to the assay.

For the determination of enzyme activities, cells were homogenised with a *Potter* homogeniser for 1 min at 1000 min^{-1} at 0 °C.

Succinate dehydrogenase was determined according to *Kremser* et al. (21). Enoyl-CoA hydratase : 3-hydroxyacyl CoA dehydrogenase (peroxisomal bifunctional enzyme) was estimated by measuring the crotonoyl-CoA dependent formation of NADH at 340 nm (22). The assay of thiolase was performed by measuring the thioester absorption band at 233 nm using acetoacetyl-CoA as a substrate (23). Catalase was assayed according to *Baudhuin* et al. (24). The protein content in cell homogenates was measured according to the method of *Bradford* (25) using the Bio-Rad protein assay kit (Bio-

Rad, Hercules, USA). All specific activities are expressed as mU/mg protein.

Preparation of RNA

Total cellular RNA was prepared according to the method described by *Sambrook* et al. (26) from confluent HepG2 and MH1C1 cells treated with the substances indicated. The RNA content was estimated by measuring the absorbance at 260 nm. Total RNA was precipitated with ethanol and stored at −70 °C.

Poly (A^+) RNA was isolated using the PolyATtract mRNA isolation system III of Promega according to the manufacturer's instructions (Promega; Madison, USA).

Northern- and slot-blot analysis

RNA was separated by electrophoresis on 1% agarose gels under denaturing conditions and then transferred to Hybond-N membranes (Amersham; Little Chalfont, UK) by capillary blotting technique (26). RNA molecular weight marker II (Boehringer Mannheim; Mannheim, Germany) was used as size standard in RNA gel electrophoresis.

For slot-blot analysis total RNA was denatured with formaldehyde and applied to Hybond-N membranes. RNA was fixed to the membranes by UV-crosslinking. The probes used in the hybridisation procedures included restriction fragments of *Pst*I fragment of rat acyl CoA oxidase (686 bases) (27), *Xba*I fragment of human enoyl-CoA hydratase : 3-hydroxyacyl-CoA dehydrogenase (1×10^3 bases) (28), *Eco*RI fragment of human catalase (2.2×10^3 bases, personal communication; Prof. *I. Singh*), *Pst*I fragment of rat catalase (1×10^3 bases) (29), *Eco*RI fragment of human β-actin (1.1×10^3 bases) (30) and *Pst*I fragment of mouse β-actin (1.1×10^3 bases) (31).

cDNA probes were labelled with [α-^{32}P]dCTP using the NEBlot Kit (New England Biolabs; Beverly, USA) (32). Membranes were prehybridised in a solution containing 500 g/l formamide, 0.75 mol/l NaCl, 75 mmol/l sodium citrate (pH = 7), 0.4 g/l Ficoll 400, 0.4 g/l polyvinylpyrrolidone, 0.4 g/l bovine serum albumin (Fraction V), 1 g/l SDS, 200 mg/l salmon sperm DNA and 100 g/l dextran sulphate at 42 °C for 2 h and after addition of the denatured probes hybridisation was carried out overnight. Membranes were washed twice for 15 min in 0.3 mol/l NaCl, 30 mmol/l sodium citrate (pH = 7), 1 g/l SDS, at room temperature, followed by two washes for 15 min at 42 °C in 0.15 mol/l NaCl, 15 mmol/l sodium citrate (pH = 7), 1 g/l SDS. Membranes were autoradiographed using Kodak X-Omat films and intensifying screens (Kodak; Rochester, USA).

After autoradiography, the RNA filters were washed using 0.005 mol/l Tris-HCl (pH 8.0), 0.002 mol/l EDTA and 0.02 g/l Ficoll 400, 0.02 g/l polyvinylpyrrolidone, 0.02 g/l bovine serum albumin (Fraction V), 0.05 g/l SDS for 2 h at 65 °C and reprobed. Membranes treated this way revealed no signs of diminished quality after reprobing. Human and mouse β-actin cDNA were used to rehybridise the membranes and served as an internal standard for the normalisation of the RNA levels. Slot blots were quantified by counting each blot with the InstantImager (Canberra Packard; Meriden, USA), and densitometry was performed using the Pharmacia Image Master (Pharmacia; Uppsala, Sweden).

Cytotoxicity assay

Cytotoxicity was assessed by measurement of the activity of mitochondrial dehydrogenases of viable cells (33). Cells were seeded on 1 ml microtitration plates at cell densities of 10×10^6/l, 50×10^6/l, and 100×10^6/l. Twentyfour hours after plating cells were treated with the test compounds for 24 hours. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) was added directly to the wells. After 3 hours at 37 °C media was removed, the formazan was solubilized in dimethylsulphoxide/acetic acid/SDS (100 g/l SDS, 100 mmol/l acetic acid in dimethylsulphoxide) and the plates were read on a Microplate Autoreader (Bio-Tek Instruments, Winooski, USA) at 570 nm. The formazan is produced proportional to the dehydrogenase activity.

Morphology

After treatment with the test compound cells were harvested and washed twice with ice-cold phosphate buffered saline and fixed in ice-cold glutaraldehyde (25 g/l) in 0.2 mol/l phosphate buffer, pH 7.2. After 3 hours of fixation the cells were washed with phosphate buffered saline and postfixation with 1% osmium tetroxide was employed. The specimens were washed in distilled water, dehydrated in a series of graded ethanol solutions and routinely embedded in Epon 812. Ultrathin section were cut with an ultramicrotome (Reichert, Vienna, Austria) and examined in a Zeiss EM9S/2 electron microscope (Zeiss, Oberkochen, Germany).

Statistics

Statistical comparison between the control groups and the treated groups were made, using analysis of variance followed by *Student*'s t-test.

Results

Morphology

Electron microscopical comparisons of hepatoma cell lines (treated and untreated) were carried out to confirm proliferation by the application of clofibric acid. HepG2 cells presented inconspicuously. They were provided with abundant free ribosomes, contained short segments of rough endoplasmic reticulum, *Golgi* areas and cristae-type mitochondria (fig. 1). Peroxisomes were rarely identified. These organelles were mostly oval in shape

Fig. 1 Electron micrographs showing peroxisomes in HepG2 cells, control preparation with dimethylsulphoxide. Cells contain abundant ribosomes, segments of rough endoplasmic reticulum, *Golgi* regions, mitochondria, and a few lipid droplets. Microbodies are rarely seen (inserts); they represent microperoxisomes with granular matrix but without a crystalline core. Magnification: × 1900; insert × 8900.

and measured 0.2–0.25 µm in diameter. They contained granular matrix material of moderate electron density and were confined by a single membrane (fig. 1, inserts). After treatment with clofibric acid, the number of microperoxisomal profiles was increased but morphology and size of the individual organelles appeared unchanged (fig. 2).

Comparable results were obtained with the rat hepatoma cell line MH1C1 (results not shown).

Cytotoxicity

The toxicological effects of the compounds used were verified by measurement of the mitochondrial dehydrogenases of the cells in order to estimate the concentration range of cytotoxicity, because toxicological effects need to be avoided for proliferation experiments. The results of the cytotoxicity assays indicate that clofibric acid and triiodothyronine do not influence the viability of both cell lines at concentrations below 5 mmol/l and 100 µmol/l, respectively (data not shown). Cetaben treated cells already show a significant response at 10 µmol/l. The fractional survivals of HepG2 and MH1C1 cell lines at a number of 100 000 cells per well treated with 100 µmol/l cetaben were 66% and 45% of control ($p < 0.001$) respectively (fig. 3: A–B). The cytotoxic effect was more pronounced at a cell number of 10 000, where the fractional survival for both cell lines was 37% ($p < 0.001$). The rat hepatoma cell line showed a greater sensitivity at higher cell number towards cetaben than the human cell line (fig. 3: A–B). Since cytotoxicity was lower at higher cell densities confluent monolayers were used for all experiments. These findings agree with previous morphological studies on rat hepatocytes (34).

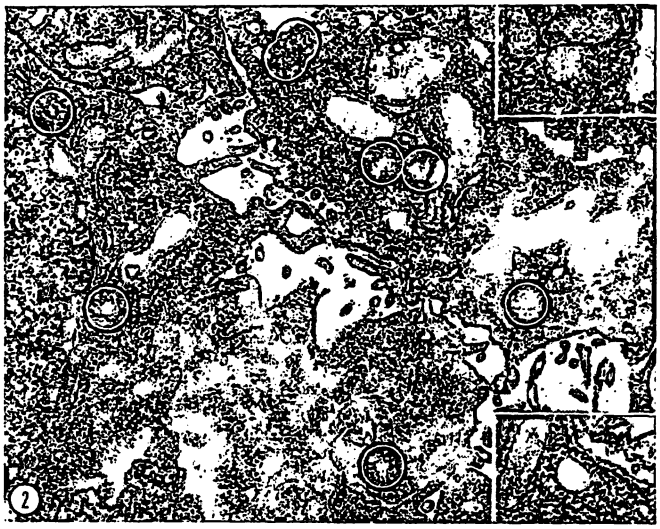
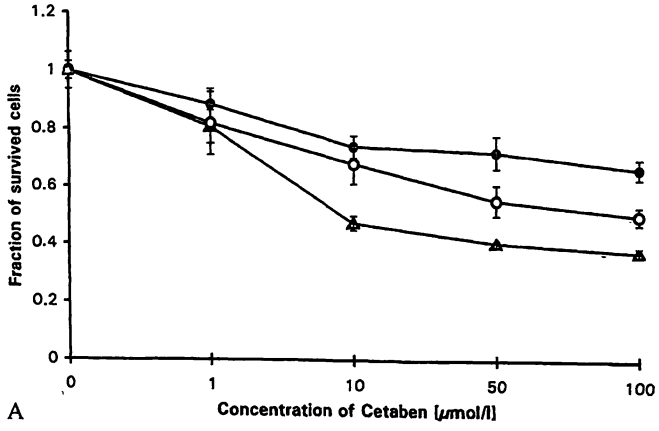


Fig. 2 Electron micrographs showing peroxisomes in HepG2 cells, cells treated with 3 mmol/l clofibric acid. Gross morphology of HepG2 cells closely resembles that of control cells (compare with fig. 1). The number of microperoxisomes, however, is increased (encircled regions; two of them are shown at higher magnification in the inserts). Note that mitochondrial volume also appears slightly enlarged. Magnification: × 1900; insert × 8900.

Enzymes

In order to quantify the difference of the induction of peroxisomal proliferation between human (HepG2) and rat (MH1C1) cells, peroxisomal and mitochondrial enzymatic activities were determined.

In HepG2 3 mmol/l clofibric acid had no effect on thiolase and the peroxisomal bifunctional enzyme activities. The activity of catalase was increased from 19.9 ± 2.1 to 26.7 ± 2.7 mU/mg ($p < 0.01$), succinate dehydrogenase activity from 13.5 ± 0.7 to 18 ± 2.5 mU/mg by 3 mmol/l clofibric acid (see tab. 1A). Catalase activity was increased by 10 µmol/l and 100 µmol/l cetaben to about 120% of control ($p < 0.01$; control activity: 17.7 ± 1.2 mU/mg), whereas the peroxisomal bifunctional enzyme was not affected (control activity: 37.9 ± 4.4 mU/mg). Ten µmol/l cetaben did not alter the activity



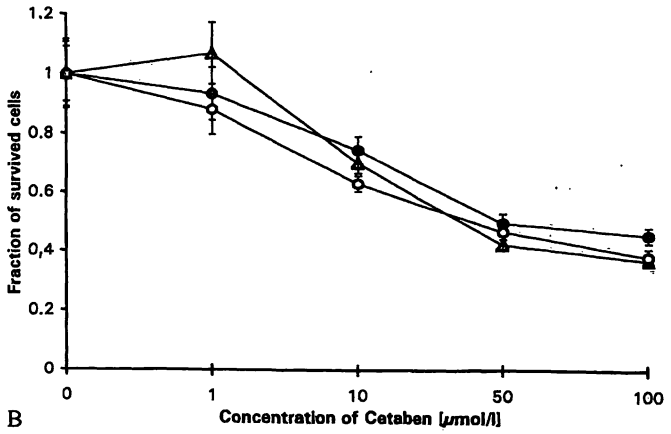


Fig. 3A–B Comparison of the dose response of HepG2 and MH1C1 cell lines at different concentrations of cetaben. Fractional survival of HepG2 (Panel A) and MH1C1 (Panel B) using the cytotoxicity assay (see Method's section). Filled circles: cell number = 100.000; open circles: cell number = 50.000; open triangles: cell number = 10.000. All experiments were performed in triplicate.

of succinate dehydrogenase, whereas the activity was decreased by 100 µmol/l cetaben from 11.8 ± 1.0 to 8.7 ± 1.4 mU/mg ($p < 0.01$). Thiolase activity was slightly enhanced by 10 µmol/l cetaben from 319.5 ± 43.6 to 379.3 ± 30.1 mU/mg, but 100 µmol/l cetaben slightly decreased the activity to 283.6 ± 35 mU/mg (tab. 1A). In HepG2 cells the activities of all enzymes were not affected by treatment with 10 µmol/l and 100 µmol/l triiodothyronine (data not shown).

Tab. 1 Relative specific activities of catalase, the peroxisomal bifunctional enzyme, succinate dehydrogenase and thiolase of HepG2 (Panel A) and MH1C1 (Panel B) cells after treatment for 24 hours with the indicated compounds.

Compound	Clofibric acid 3 mmol/l	Cetaben 10 µmol/l	Cetaben 100 µmol/l
A: HepG2 cells			
Catalase	135 ± 10* n = 5	119 ± 3* n = 4	118 ± 8* n = 7
Thiolase	96 ± 5 n = 4	119 ± 8 n = 4	89 ± 12 n = 4
Bifunctional enzyme	116 ± 12 n = 4	97 ± 7 n = 5	96 ± 12 n = 5
Succinate dehydrogenase	133 ± 14** n = 4	110 ± 17 n = 5	75 ± 16* n = 5
B: MH1C1 cells			
Catalase	104 ± 8 n = 4	104 ± 10 n = 4	106 ± 11 n = 4
Thiolase	125 ± 8 n = 3	129 ± 8** n = 3	123 ± 14 n = 3
Bifunctional enzyme	112 ± 25 n = 4	89 ± 19 n = 3	95 ± 4 n = 4
Succinate dehydrogenase	118 ± 30 n = 4	75 ± 10 n = 3	57 ± 16* n = 4

The values are given as % of control ± %SEM. * $p < 0.01$; ** $p < 0.02$; n indicates the number of experiments. The specific activities are given in the result section.

Three mmol/l clofibric acid did not increase the activities of catalase (control activity 71.1 ± 1.9 mU/mg), succinate dehydrogenase (control activity 7.1 ± 2.5 mU/mg) and the peroxisomal bifunctional enzyme (control activity 52.9 ± 5.4 mU/mg) in MH1C1 cells, whereas thiolase activity was enhanced from 166.5 ± 21.9 to 207.6 ± 12.5 mU/mg (tab. 1B). The mitochondrial marker succinate dehydrogenase was decreased by 10 µmol/l cetaben from 8.9 ± 1.2 to 6.7 ± 0.7 mU/mg and further significantly decreased by 100 µmol/l cetaben to 5.1 ± 0.8 mU/mg ($p < 0.01$) in MH1C1. Thiolase activity was enhanced by 10 µmol/l and 100 µmol/l cetaben from 172.4 ± 8.2 to 222.2 ± 18.6 mU/mg ($p < 0.02$) and to 212.8 ± 30.1 mU/mg. Catalase and the peroxisomal bifunctional enzyme showed no response to the treatment with cetaben in MH1C1 cells (tab. 1B). The activities of all enzymes were not significantly affected by treatment with 10 µmol/l and 100 µmol/l triiodothyronine in MH1C1 cells (data not shown).

RNA analysis

Northern blot experiments with mRNA of HepG2 (fig. 4) and MH1C1 (fig. 5) were carried out to confirm the identity of the probes.

In order to determine whether the observed variations in catalase, peroxisomal bifunctional enzyme and acyl-CoA oxidase activities resulted from increased expres-

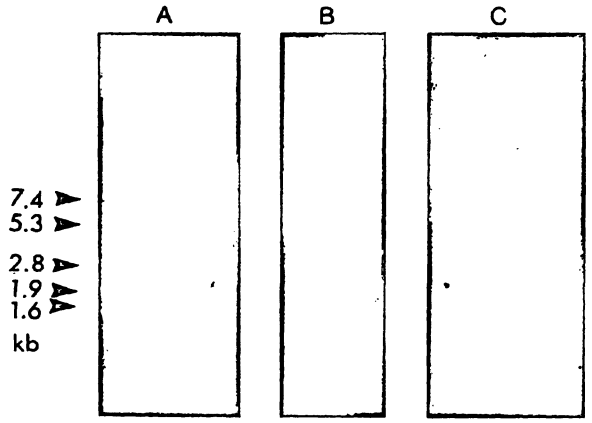


Fig. 4 Northern blot analysis of HepG2 cells after hybridisation with a cDNA probe for human β-actin (panel A), a cDNA probe for human catalase (panel B) and a probe derived from the human peroxisomal bifunctional enzyme cDNA (panel C). RNA size marker is indicated on the left. The mRNA sizes are: β-actin 2.1 × 10^3 bases, catalase 2.7 × 10^3 bases and peroxisomal bifunctional enzyme 4.6 × 10^3 bases.

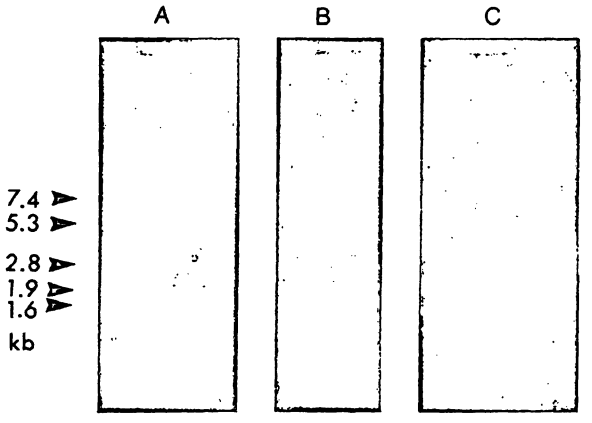


Fig. 5 Northern blot analysis of MH1C1 cells after hybridisation with a cDNA probe for mouse β-actin (panel A), a cDNA probe for rat catalase (panel B) and a cDNA probe for rat fatty acyl-CoA oxidase (panel C). RNA size marker is indicated on the left. The mRNA sizes are: catalase 2.8 × 10^3 bases, fatty acyl-CoA oxidase 4.2 × 10^3 bases and β-actin 2.2 × 10^3 bases.

sion of the corresponding genes, we determined the cellular mRNA levels after 24 hours of incubation of HepG2 and MH1C1 cells with 3 mmol/l clofibric acid, 10 µmol/l and 100 µmol/l cetaben with different amounts of RNA. Cell monolayers from the corresponding subcultures, which were used for determination of enzyme activities or cytotoxicity assays, were incubated in parallel in *Dulbecco*'s Modified Eagles medium with or without peroxisomal proliferators. mRNA levels were assayed by slot-blotting, using specific ^{32}P-labelled probes (fig. 6, 7 and 8), as described in Materials and Methods. The β-actin mRNA level was used as an internal standard.

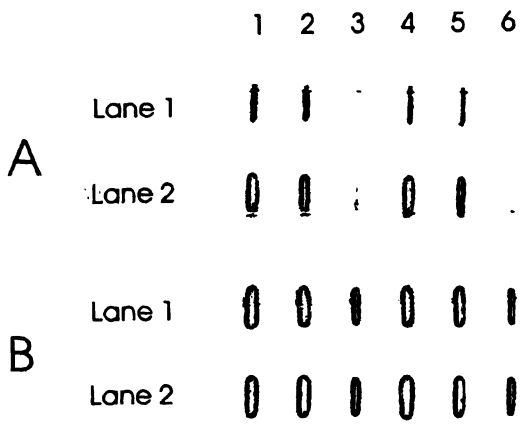


Fig. 6 Slot blot analysis of the effect of clofibric acid on catalase in HepG2 cells. HepG2 cells were exposed to 3 mmol/l clofibric acid for 24 h. Total RNA was purified, denatured and applied to the membrane (slots 1, 4: 20 µg; slots 2, 5: 10 µg; slots 3, 6: 1 µg; lane 1: control cells; lane 2: 3 mmol/l clofibric acid). The membrane was hybridised with ^{32}P-labelled cDNAs for catalase (panel A), washed and rehybridised with a probe for β-actin (panel B).

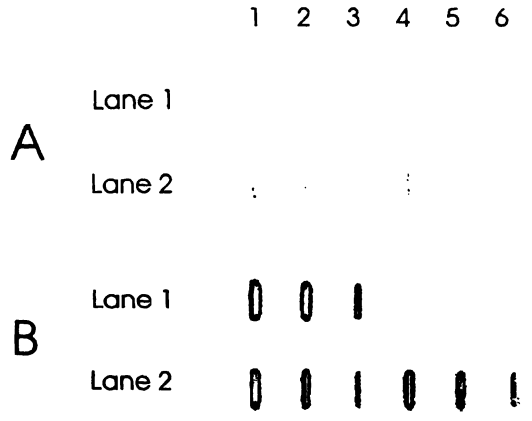


Fig. 7 Slot blot analysis of the effect of cetaben on the peroxidase bifunctional enzyme in HepG2 cells. HepG2 cells were exposed to 10 µmol/l (slots 1, 2, 3) and 100 µmol/l (slots 4, 5, 6) cetaben for 24 h. Total RNA was purified, denatured and applied to the membrane; lane 1: control cells (slot 1: 20 µg; slot 2: 10 µg; slot 3: 1 µg); lane 2: Cetaben (slots 1, 4: 20 µg; slots 2, 5: 10 µg; slots 3, 6: 1 µg). The membrane was hybridised with ^{32}P-labelled cDNAs for peroxisomal bifunctional enzyme (panel A), washed and rehybridised with a probe for β-actin (panel B).

As shown in table 2 catalase mRNA was 2.6 fold higher in HepG2 cells treated with 3 mmol/l clofibric acid than in controls ($p < 0.001$). The significant increase of mRNA levels induced by clofibric acid also correlates with the higher activity of catalase (tab. 1). Interestingly the mRNA levels of the peroxisomal bifunctional enzyme were not increased by 3 mmol/l clofibric acid

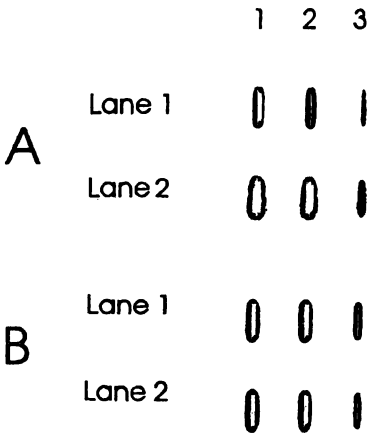


Fig. 8 Slot blot analysis of the effect of clofibric acid on fatty acyl-CoA oxidase in MH1C1 cells. MH1C1 cells were exposed to 3 mmol/l clofibric acid for 24 h. Total RNA was purified, denatured and applied to the membrane (slot 1: 20 µg; slot 2: 10 µg; slot 3: 1 µg; lane 1: control cells; lane 2: 3 mmol/l clofibric acid). The membrane was hybridised with ^{32}P-labelled cDNAs for fatty acyl-CoA oxidase (panel A), washed and rehybridised with a probe for β-actin (panel B).

Tab. 2 Relative mRNA levels of catalase and the peroxisomal bifunctional enzyme in HepG2 cells and of catalase and fatty acyl-CoA oxidase mRNAs in MH1C1 cells after treatment for 24 h.

Cell line	Treatment	Relative mRNA values	
		human catalase	human peroxisomal bifunctional enzyme
HepG2	Clofibric acid, 3 mmol/l	2.6 ± 0.22**	1.0 ± 0.15
	Cetaben, 10 µmol/l		2.3 ± 0.14*
	Cetaben, 100 µmol/l		1.4 ± 0.14
		rat catalase	rat fatty acyl-CoA oxidase
MH1C1	Clofibric acid, 3 mmol/l	0.9 ± 0.06	1.8 ± 0.29**
	Cetaben, 100 µmol/l		2.4 ± 0.64*

After autoradiography, the densities were recorded and expressed in conventional densitometric units to calculate the relative levels. Each value was divided by the corresponding mRNA β-actin value. Finally, the relative levels of mRNA in treated cells were divided by the relative values of parallel non-treated control cells. The ratios are given as mean ± SEM; n = 3; * $p < 0.01$, ** $p < 0.001$ as compared with the corresponding control value.

which correlates well with the enzyme activities in HepG2 (tab. 1).

The mRNA level for the peroxisomal bifunctional enzyme in HepG2 cells treated with 10 µmol/l cetaben was elevated significantly to 2.3 fold as compared with the control ($p < 0.01$), whereas treatment with 100 µmol/l cetaben did not cause a significant increase. These findings do not correlate with the enzymatic activities of the peroxisomal bifunctional enzyme which remained unchanged at all concentrations of cetaben.

The mRNA for fatty acyl-CoA oxidase of MH1C1 cells was increased 1.8 fold ($p < 0.001$) by 3 mmol/l clofibric acid which correlates well with the findings of *Thangada* et al. (34) using Ciprofibrate in primary hepatocyte cultures. Cetaben treated cells showed a similar increase in fatty acyl-CoA oxidase (2.4 fold, $p < 0.01$) using 100 µmol/l cetaben (tab. 2).

Catalase mRNA was not affected by 3 mmol/l clofibric acid (tab. 2) which parallels the enzymatic activity.

Discussion

The main objective of the present study was to show the difference in the regulation of the peroxisomal β-oxidation of a human (HepG2) and rat (MH1C1) cell line. In order to elucidate if the effects on the peroxisomal β-oxidation are correlated with the cytotoxicity of the chemicals a cytotoxicity assay was carried out. This assay indicates that clofibric acid and triiodothyronine at concentration of 5 mmol/l and 100 µmol/l, respectively, are not toxic, whereas cetaben is highly toxic even at low concentrations (100 µmol/l). Therefore the effects of higher concentrations of cetaben on the peroxisomal β-oxidation are superimposed by its cytotoxicity. Succinate dehydrogenase, a mitochondrial marker enzyme, was used to check the status of the mitochondrial energy producing system to verify the energy state of the cells. Clofibric acid enhances succinate dehydrogenase whereas cetaben, due to its cytotoxicity, significantly decreases its activity (see tab. 1). This differential effect is more pronounced in MH1C1 cells indicating that rat cells are more sensitive to peroxisomal proliferators. On the other hand the activity of catalase, a peroxisomal marker, is increased in HepG2 cells by cetaben and clofibric acid, which is caused by a much higher response of mRNA synthesis (see tab. 1 and 2), and additionally the response of mRNA by clofibric acid treated cells is much higher than the increase in activity. Moreover the activities of the peroxisomal bifunctional enzyme in both cell lines are unaffected, although the mRNA level was enhanced to 2.3 fold by 10 µmol/l cetaben in HepG2 cells. In order to elucidate whether the increase of the activities of peroxisomal enzymes in hepatoma cell lines is weaker than in primary hepatocytes (34) or whether the increase is delayed, as suggested by the higher mRNA levels, long term studies have to be carried out.

It seems that the proliferation of the peroxisomal bifunctional enzyme is more pronounced if compared to other enzymes like succinate dehydrogenase (at 10 µmol/l cetaben enzyme activity is 75% of control).

Interestingly the response of thiolase to clofibric acid and cetaben is higher in MH1C1 cells whereas the response in HepG2 varies. The decrease of the activity of thiolase at 100 µmol/l cetaben seems to be due to the high cytotoxicity. Cetaben also increases the activity of the peroxisomal β-oxidation system but this effect is compensated by the cytotoxicity of the compound.

At the concentrations used triiodothyronine does not trigger any response at all, which can be explained by the difference in the receptor mediated response (12). The effect of hypolipidaemic drugs seen in our cell model is less pronounced than in rat liver but stronger in the rat cell line MH1C1 than in the human cell line HepG2, but is in accordance with previous findings of *Graham* et al. (20) in primates and of *Hanefeld* et al. (35) in humans which indicates that the peroxisomal proliferation in human and primate is not as pronounced as in rat, although the lipid lowering effect is present in human (5). The hypotriglyceridaemic action of the se drugs is reported to be due to the decrease of apolipoprotein C-III gene expression, which was demonstrated by *Staels* et al. (36) in rat and human hepatocytes. Therefore this weak response of HepG2 to peroxisomal proliferators is in accordance with the assumed effect in human liver in vivo. Similar findings using human and rat hepatic cell lines have been previously reported by *Scotto* et al. (37) using clofibrate and *Brocard* et al. (38) using ciprofibrate.

Our results indicate that the human hepatoma cell line HepG2, as previously indicated by *Watkins* et al. (39), is an appropriate model for studies of the influence of hypolipidaemic agents on the peroxisomal enzyme system.

Acknowledgements

The authors would like to thank Ms. *Maria Pedru* for excellent technical assistance. Cetaben was kindly provided by American Cyanamid Company, Lederle Laboratories Division, Pearl River, USA. The cDNA for the rat acyl-CoA oxidase was a gracious gift from Prof. *T. Osumi*, the cDNA for the rat catalase was kindly

provided by Dr. *S. Furuta*. The authors would like to thank Dr. *G. Höfler* for his generous gift of cDNA for the human peroxisomal bifunctional enzyme. The cDNA for human catalase was donated by Prof. *I. Singh*.

References

1. Lazarow PB, Fujiki F. Biogenesis of peroxisomes. Ann Rev Cell Biol 1985; 1:489–530.
2. Kramar R. Die Beteiligung der Peroxisomen am Lipidstoffwechsel. J Clin Chem Clin Biochem 1986; 24:109–18.
3. Reddy JK, Mannaerts GP. Peroxisomal lipid metabolism. Annu Rev Nutr 1994; 14:343–70.
4. Sirtori CR, Galli C, Franceschini G. Fraudulent (and non fraudulent) fatty acids for human health. Eur J Clin Invest 1993; 23:686–9.
5. Sirtori CR, Manzoni C, Lovati MR. Mechanisms of lipid-lowering agents. Cardiology 1991; 78:226–35.
6. Hawkins JM, Jones WE, Bonner FW, Gibson GG. The effect of peroxisome proliferators on microsomal, peroxisomal, and mitochondrial enzyme activities in the liver and kidney. Drug Metabol Rev 1987; 18:441–515.
7. Fort FL, Stein HH, Langenberg K, Lewkowski JP, Heyman IA, Kesterson JW. Cetaben versus clofibrate comparison of toxicity and peroxisome proliferation in rats. Toxicology 1983; 28:305–11.
8. Chandoga J, Hampl L, Turecky L, Rojekova I, Uhlikova E, Hocman G. Cetaben is an exceptional type of peroxisome proliferator. Int J Biochem 1994; 26:679–96.
9. Just WW, Hartl FU, Schimassek H. Rat liver peroxisomes. I. New peroxisome population induced by thyroid hormones in the liver of male rats. Eur J Cell Biol 1982; 26:249–54.
10. Kramar R, Kremser K, Hohenegger M, Mayer M. Fatty acyl-CoA oxidase in rat kidney and liver after application of thyroxine. Enzyme 1986; 35:27–33.
11. Hryb DJ, Hoff JF. Chain length specificities of peroxisomal and mitochondrial β-oxidation in rat liver. Biochem Biophys Res Comm 1979; 87:1200–6.
12. Meier-Heusler SC, Zhu X, Juge-Aubry C, Pernin A, Burger AG, Cheng SY, Meier CA. Modulation of thyroid hormone action by mutant thyroid hormone receptors, c-erbAα2 and peroxisome proliferator-activated receptor: evidence for different mechanisms of inhibition. Mol Cell Endocrinol 1995; 107:55–66.
13. Van den Bosch H, Schutgens RBH, Wanders RJA, Tager JM. Biochemistry of peroxisomes. Annu Rev Biochem 1992; 61:157–97.
14. Osumi T, Ishii N, Miyazawa S, Hashimoto T. Isolation and structural characterization of the rat acyl-CoA oxidase gene. J Biol Chem 1987; 262:8138–43.
15. Schepers L, Van Veldhoven PP, Casteels M, Eyssen HJ, Mannaerts GP. Presence of three acyl-CoA oxidases in rat liver peroxisomes. An inducible fatty acyl-CoA oxidase, a noninducible fatty acyl-CoA oxidase, and a noninducible trihydroxycoprostanoyl-CoA oxidase. J Biol Chem 1990; 265:5242–6.
16. Ishii N, Hijikata M, Osumi T, Hashimoto T. Structural organization of the gene for rat enoyl-CoA hydratase: 3-hydroxyacyl-CoA dehydrogenase bifunctional enzyme. J Biol Chem 1987; 262:8144–50.
17. Hijikata M, Wen JK, Osumi T, Hashimoto T. Rat peroxisomal 3-ketoacyl-CoA thiolase gene. Occurrence of two closely related but differentially regulated genes. J Biol Chem 1990; 265:4600–6.
18. Bout A, Franse MM, Collins J, Blonden L, Tager JM, Benne R. Characterization of the gene encoding human peroxisomal 3-oxoacyl-CoA thiolase (ACAA). No large DNA rearrangement in a thiolase-deficient patient. Biochim Biophys Acta 1991; 1090:43–51.
19. Casteels M, Schepers L, Van Veldhoven P, Eyssen HJ, Mannaerts GP. Separate peroxisomal oxidases for fatty acyl-CoAs and trihydroxycoprostanoyl-CoA in human liver. J Lipid Res 1990; 31:1865–72.
20. Graham MJ, Wilson SA, Winham MA, Spencer AJ, Rees JA, Old SL, et al. Lack of peroxisome proliferation in marmorset liver following treatment with ciprofibrate for 3 years. Fundam Appl Toxicol 1994; 22:58–64.
21. Kremser K, Schön HJ, Lohninger A, Prager CM, Kramar R, Böck P. Response to thyroxine of lamellar bodies, peroxisomes and peroxisomal enzymes in the adult rat lung. Eur J Clin Chem Clin Biochem 1991; 29:151–8.
22. Lazarow PB. Different chain length specificities of peroxisomal and mitochondrial enoyl-CoA hydratases. Arch Biochem Biophys 1981; 206:342–5.
23. Lazarow PB. Rat liver peroxisomes catalyze the β-oxidation of fatty acids. J Biol Chem 1978; 253:1522–8.
24. Baudhuin P, Beaufay H, Rahman-Li Y, Sellinger OZ, Wattiqux R, Jacques P, et al. Tissue fractionation studies: intracellular distribution of monoamine oxidase, aspartate aminotransferase, alanine aminotransferase, D-amino acid oxidase and catalase in rat-liver tissue. Biochem J 1964; 92:169–84.
25. Bradford M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Anal Biochem 1976; 72:248–54.
26. Sambrook J, Fritsch EF, Maniatis T. Molecular cloning. A laboratory manual. 2nd ed. Cold Spring Habor, New York: Cold Spring Harbor Laboratory, 1989.
27. Miyazawa S, Osumi T, Hashimoto T, Ohno K, Miura S, Sujiki Y. Peroxisome targeting signal of rat liver acyl-coenzyme A oxidase resides at the carboxy terminus. Mol Cell Biol 1989; 9:83–91.
28. Hoefler G, Forstner M, McGuinness MC, Hulla W, Hiden M, Krisper P, et al. cDNA cloning of the human peroxisomal enoyl-CoA hydratase : 3-Hydroxyacyl-CoA dehydrogenase bifunctional enzyme and localization to chromosome 3q26.3–3q28: a free left *Alu* arm is inserted in the 3'noncoding region. Genomics 1994; 19:60–7.
29. Furuta S, Hayashi H, Hijikata M, Miyazawa S, Osumi T, Hashimoto T. Complete nucleotide sequence of cDNA and deduced amino acid sequence of rat liver catalase. Proc Natl Acad Sci USA 1986; 83:313–7.
30. Adams MD, Kelley JM, Gocayne JD, Dubnick M, Polymeropoulos MH, Xiao H, et al. Complementary DNA sequencing: expressed sequence tags and human genome project. Science 1991; 252:1651–6.
31. Minty AJ, Alonso S, Guenet JL, Buckingham ME. Number of organization of actin-related sequences in the mouse genome. J Mol Biol 1983; 167:77–101.
32. Feinberg AP, Vogelstein B. A technique for radiolabelling DNA restriction endonuclease fragments to high specificity. Anal Biochem 1983; 132:6–13.
33. Mossman T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Meth 1983; 65:55–63.
34. Thangada S, Alvares K, Mangino M, Usman MI, Rao MS, Reddy JK. An in vitro demonstration of peroxisomes proliferation and increase in peroxisomal β-oxidation system mRNAs in cultured rat hepatocytes treated with ciprofibrate. FEBS Letters 1989; 250:205–10.
35. Hanefeld M, Kemmer C, Kadner E. Relationship between morphological changes and lipid-lowering action of p-chlorphenoxyisobutyric acid (CPIB) on hepatic mitochondria and peroxisomes in man. Atheriosclerosis 1983; 46:239–46.
36. Staels B, Vu-Dac N, Kosykh VA, Saladin R, Fruchart JC, Dallongeville J, Auwerx J. Fibrates downregulate apolipoprotein C-III expression independent of induction of peroxisomal acyl coenzyme A oxidase. J Clin Invest 1995; 95:705–12.
37. Scotto C, Keller JM, Schohn H, Dauca M. Comparative effects of clofibrate on peroxisomal enzymes of human (Hep EBNA2)

and rat (FaO) hepatoma cell lines. Eur J Cell Biol 1995; 66:375–81.
38. Brocard C, Es-Souni M, Ramirez LC. Latruffe N, Bournot P. Stimulation of peroxisomal palmitoyl-CoA oxidase activity by ciprofibrate in hepatic cell lines: comparative studies in Fao, MH1C1 and HepG2 cells. Biol Cell 1993; 77:37–41.
39. Watkins PA, Ferrell EV, Pedersen JI, Hoefler G. Peroxisomal fatty acid β-oxidation in HepG2 cells. Arch Biochem Biophys 1991; 289:329–36.

Dr. Karl Kremser
Institut für Medizinische Chemie
der Universität Wien
Währingerstraße 10
A-1090 Wien
Austria